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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2020 17(4): 4034-4044. **OPEN ACCESS**

The Anti-proliferative effect of *Ficus Carica* and *Olea Europaea* leaves extracts involves apoptosis and cell cycle regulation in hepg2 cells

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Hepatocellular carcinoma (HCC) is a common and aggressive malignant tumor with a poorly defined molecular mechanisms. HepG2 cells were treated with different concentrations of *F. carica* and *O. europaea* leaves extracts to determine their effect on cell viability through MTT assay. Gene expression of *Cyclin D1*, *Cdk2* and *Bcl-2* was detected by real time PCR. Flow cytometer was used for cell cycle and apoptosis analysis. Results of MTT assay indicated that *F. carica* and *O. europaea* inhibited HepG2 cell proliferation in a dose and time dependent manner. Treatment with *F. carica* and *O. europaea* down-regulate Cyclin D1 and CDK2 gene expression. Induction of apoptosis was revealed to the inhibition of Bcl-2 expression and the increase of apoptotic cells. F. carica and O. europaea showed antitumor activity on HepG2 cells. O. europaea showed more potential activity than F. carica leaves extract.

Keywords: Hepatocellular Carcinoma, Apoptosis, O. europaea, F. carica, Cyclin D1, Cdk2, Bcl-2

INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive malignancy, which considered as the third leading cause of cancer-related death worldwide. Several therapeutic approaches including radiotherapy, chemotherapy and immunotherapy are available for the management of different cancer types including hepatocellular carcinoma (Si et al. 2019). Chemotherapy is the most mutual treatment protocol for HCC treatment (Rinninella et al. 2107). Triggering of apoptotic death (type 1 cell death), is a conservative policy that mediated the antitumor activity of most chemotherapeutic agents (El-Shafey and Elsherbiny, 2019a, b; El-Shafey, 2019). Apoptosis is a well-organized type of cell death that triggered by distinct biochemical pathways. The apoptotic machinery is mediated through the communication between pro-survival and pro-apoptotic (e.g.: Bcl-2), signaling molecules (Abdel-Mohsen et al. 2017). Though, many tumors, including HCC, have developed mechanisms that stimulate resistance to apoptosis, targeting those mechanisms represent potential therapeutic strategy against tumorigenesis (Abdel-Mohsen et al. 2019a).

In the same context, the deranged expression of cell cycle related proteins (e.g.: Cyclin D1 and Cdk2), considered as a vital feature that contribute to the development of hepatocellular carcinoma and induction of drug resistance (Qie et al. 2016). Cyclin D1 is defined as a regulatory subunit of cyclin dependent kinases Cdk4 and Cdk6. It is synthesized at the G1 phase and then binds with CDK4/6 to regulate the G1/S-phase transition (Dong et al.2018). During the S phase of

cell cycle, Cyclin D1 is degraded by autophagy in the cytoplasm (Wu et al. 2018a). Initiation of HCC development is mediated by Cyclin D1 overexpression which promote the progression of cell-cycle leading to the induction of proliferation and hepato-carcinogenesis (Wu et al. 2018b; El-Shafey, 2018). Consequently, Cdk2, is one of the Cdk family that control the G1/S and G2/M checkpoints. Phosphorylation of the serine/ threonine residues on the substrate leads to the promotion of cell cycle evolution (Xu et al. 2019; Lim and Kaldis, 2013). Tumor cell hyper-proliferation is accompanied with abnormal activation of Cdk2 which lead to abnormalities in G1/S and G2/M checkpoints (Xu et al. 2019). Thus, targeting deregulated expression of these proteins specifically in malignancies could have potential therapeutic value.

The therapeutic role of polyphenol plant extracts as chemo-preventive and anti-cancer agents have been reported (Hayat et al. 2020; Hazrat et al.2020). *Ficus Carica* or Fig (*Cassia fistula*), was reported to show anti-cancerous activity and actively involved in the pathogenesis of a wide number of diseases including atherosclerosis, cardiac and cerebral ischemia and carcinogenesis (Zhao et al. 2016). The pharmacological activity of many *Ficus* species (eg: hepato-protective), could referred to their significant genetic diversity (Kushwaha and Agrawal, 2017). Moreover, the bioactive constituent found in *F. carica* including phenolic acids, chlorogenic acids, flavones, flavonoids and flavonols mediate this therapeutic activity (Badgujar et al. 2014). In the same context, *Olea Europaea* or Olive tree (*Oleaceae*), was anciently known in the Mediterranean basin (Al-Attar and Shawush, 2014). Olive leaves constitute from different water-soluble compounds that exerts antioxidant and anti-inflammatory activities (Hashmi et al. 2015). The biological activity of Olive leaves is due to the presence of oleuropein (13.4%) and rutin (0.18%), luteolin-7-glucoside, apigenin-7-glucoside, hydroxyl tyrosol, verbascoside and some other hydrolytic products (Gorzynik-Debicka et al. 2018). The leaves also contain many terpenoids which exerts hepato-protective and cancer-preventive potential activity (Madrigal-Santillán et al. 2014).

With regards to the aforementioned findings, the present study aimed to explore the possible therapeutic value of *O. Europaea* (Olive) and *F. carica* (Fig), leaves extracts in hepato-carcinoma treatment, through monitoring their activity on proliferation of HCC cells, apoptotic cell death and cell cycle regulators (Cyclin D1 and Cdk2).

MATERIALS AND METHODS

2.1. Material

2.1.1. Chemicals

The chemicals utilized in this study were of analytical grade, product of: Sigma-Aldrich (USA), Lonza (Belgium) tissue culture media and supplies chemicals, Greiner bio-one (Germany) tissue culture wears.

II.1.2. Plant Materials

F. carica leaves and *O. europaea* leaves were collected locally from their natural habitats in Egypt (Delta region).

2.2. Methods

2.2.1. Preparation of Extracts

F. carica and *O. europaea* leaves were washed several times and allowed to dry at room temperature for 3 weeks. Fine powder of *F. carica* and *O. europaea* leaves was obtained and 200 gm of pulverized material was soaked in 250 ml solvent (alcohol, Analar grade). The mix was kept at room temperature for 72 hours and stirred regularly every 24 hours. Soaked samples were filtered in a fume chamber and allowed to dry at room temperature. Extracts obtained was stored at 4°C in dark.

2.2.2. Preparations of Stock and Working Solution of Plant Extracts

Stock solution of plant extracts was prepared in a concentration of 10 µg/ ml as follow: [0.1% DMSO (di-methyl sulfoxide): PBS (Phosphate buffer saline, pH 7.2)]. Working solution of plant extracts was freshly prepared and then used in different concentrations of (100,150,200, 250 and 300 µg/ml).

2.2.3. Cell line and Culture Conditions

Hepatocellular carcinoma cell line (HepG2). was obtained from (Vacsera Co. Egypt), and routinely cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100U/ ml penicillin, 100ug/ml streptomycin, in monolayer culture and kept in the incubator in a humidified atmosphere comprising 5% CO_2 in air. As cells reached confluence, they were sub-cultured to allow more space for continued growth by Trypsin-EDTA solution. Cells for each passage were counted and tested for viability by trypan blue dye exclusion assay.

2.2.4. Study Design

When HepG2 cells reached the confluence, they were incubated with different concentrations (e.g.: 100, 150, 200, 250 and 300 µg/ ml), of Olaea *europaea* (Olive) or Ficus *carica* (Fig), leaves extracts. Different investigations were performed to monitor the cytotoxic effect of *Olaea europaea* (olive) and *Ficus carica* (Fig), leaves extracts on HepG2 cells according to the following groups:

Control group: Untreated HepG2 cells**.**
Group 1 (Fig24hr.): HepG2 cells treated with Ficus carica (Fig) for 24hr.
Group 2 (Fig48hr.): HepG2 cells treated with Ficus carica (Fig) for 48hr.
Group 3 (Fig72hr.): HepG2 cells treated with Ficus carica (Fig) for 72hr.
Group 4 (Olive 24hr.): HepG2 cells treated with Olaea europaea (Olive) for 24hr.
Group 5 (Olive 48hr.): HepG2 cells treated with Olaea europaea (Olive) for 48hr.
Group 6 (Olive 72hr.): HepG2 cells treated with Olaea europaea (Olive) for 72hr.

2.2.5. Measurement of Cell Viability (MTT Assay)

Cell viability was determined using a conventional 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) reduction assay. Cells were plated at a density of 1×10^4 cells/well in 24-well plates and maintained in DMEM containing 10% FBS. The cells were made quiescent at confluence by incubation in serum-free DMEM for 24 hours, followed by treatment with different concentrations of extracts for the desired time. After incubation, phosphate-buffered saline (PBS), was utilized for washing 3 times then cells were treated with MTT solution (final concentration, 5 mg/ml), for 4 hours at 37˚C. The supernatant formed was liquefied with DMSO (Abdel-Mohsen et al. 2017). Absorbance at 570 nm was ant was then discarded, and the crystal measured with a microplate reader (Molecular device, US/V Max).

2.2.6. Cell Cycle Analysis

HepG2 cells (1×10^6 cell/ml), were permitted to grow in a 96-well culture plate and treated according to the design of the study at 37°C for 24, 48 and 72 hours. After trypsinization, cells were gathered, washed away with phosphate-buffered saline (PBS), and then cell pellets were re-suspended in 1 ml of 50 µg/ml solution of propidium iodide (PI) in buffer. Cells were kept in the incubator at 37°C for 15 min. Distribution of cell cycle was carried out via FACS Calibur (Becton Dickinson, CA, USA), (Abdel-Mohsen et al. 2019a; El-Shafey and Elsherbiny, 2019b, c).

2.2.7. Apoptosis Determination

HepG2 cells were managed according to the design of the study and kept in the incubator at 37°C for 24, 48 and 72 hours in a humidified atmosphere of 5% CO_2 at 37°C. Treated and untreated cells were collected. Binding buffer (400 ml), was utilized for washing cells twice. An annexin V-FITC (fluorescein isothiocyanate) Kit (BD Co., MA, Ltd, USA), was used to quantify the percentage of cells subjected to apoptosis. Cells was incubated with 100 ml from the mixture (1 ml annexin V-FITC conjugate and 10 ml PI), for 15 min at 25°C (Abdel-Mohsen et al. 2019b). Quantification of apoptosis was performed utilizing BD FACS Calibur flow cytometer and data were analyzed by the BD Cell Quest™ Pro (version 5.2) software.

2.2.8. RT-PCR Quantification Assay

Total RNA was prepared using trizol reagent (Invitrogen Life Technologies). Reverse transcription was carried out for 1µg of total RNA utilizing superscript 2 RNAse H-reverse transcriptase oligo (dT), (Invitrogen Life Technologies, France). Real-time PCR was assessed using the icycler iQ™ real Time detection system (Applied). Amplification reactions were done using SYBR Green using Applied Bio-systems 7500 fast real-time PCR system, with the specific primers as previously described (Ahmed et al. 2017). The sequences of the primers are:

Cyclin D1:
Forward, 5′-GTG TAT CGA GAG GCC AAA GG-3′
Reverse, 5′-GCA ACC AGA AAT GCA CAG AC-3′;
CdK2:
Forward, 5′-CAG GAT GTG ACC AAG CCA GT-3′
Reverse, 5′-TGA GTC CAA ATA GCC CAA GG-3′; and
Bcl-2:
Forward, 5′-CCG GAT CAC CAT CTG AAG AG-3′
Reverse, 5′-AGG GCA AAG AAA TGC AAG TG-3′
GAPDH:
Forward, 5′-AGG TCC ACC ACT GAC ACG TT-3′
Reverse, 5′-GCC TCA AGA TCA TCA GCA AT-3′

2.2.9. Statistical Analysis

SPSS 22.0 for windows was used for statistical analysis. For comparison of the quantitative variables, the data attained from three independent experiments (triplicate), were used. Data were presented as Mean ± SEM. Comparisons of means were done using appropriate post hoc tests of analysis of variance. Values of *P* were considered statistically significant at level ≤ 0.05.

RESULTS

3.1. Anti-proliferation Using MTT assay

Anticancer activity was evaluated using an MTT in vitro cell proliferation assay, which is a general anticancer activity evaluation test. Based on the MTT assay absorbance, treatment with *Ficus carica* extract (Fig), significantly inhibits the growth of HepG2 cells in a dose and time-dependent manner (Figure 1.a). *F carica* leaves extract showed IC_{50} (239.19, 209.34 and 172.09 μg/ml), after treatment for (24, 48 and 72 hours; respectively) (Table 1). The effective anti-proliferation activity of *F. carica*, extract was shown after treatment for 72 hr.

Table 1: IC_{50} concentrations *of F. carica* and O. *europaea* extracts used in in Hepatocellular carcinoma cell line (HepG2) treated groups.

Treated groups	IC_{50} concentrations (µg/ml)	
	***F. carica* treated cells**	***O. europaea* treated cells**
24 hr. treatment	239.19	198.83
28 hr. treatment	209.34	187.43
72 hr. treatment	172.09	152.27

In the same context, treatment with *Olaea europaea* extract significantly inhibits the growth of HepG2 cells in a dose and time-dependent manner. *O. europaea* leaves extract showed IC_{50} values (198.83, 187.43 and 152.27 µg/ml), after treatment for (24, 48 and 72 hours; respectively**)** (Table 1). The higher inhibitory effect of *Olaea europaea extract* was shown after treatment for 72 hr. (Figure 1.b). The anti-proliferation activity of O. *europaea* extract was more potent than the effect of *F. carica* extract after 24, 48 and 72 hr. (Figure 1.c).

3.2. Cell Cycle kinetics

Analysis of untreated HepG2 cells by flow cytometry (Figure 2), showed the presence of 3.4% of cells at sub G population, 51.6 % of cells distributed in G0/G1 ,29.6 % in S phase and 15.6% in G2/M phase of the cell cycle. Treatment of HepG2 cells with IC_{50} concentration of *F. carica* for 24 hour caused slight increase of cells in sub G1 fraction (4.7 %), and induced cycle arrest in G0/G1 phase (69.1 %). Treatment of HepG2 cells with IC_{50} concentration of *F. carica* for 48 hour caused increase of cells to 9.4% in sub G peak and increase of cells in the G2/M phase compared to untreated cells. While, treatment with IC_{50} concentration of *F. carica* for 72 hour caused accumulation of cells by 19.7% in sub G peak and most of cells were accumulated in G0/G1 phase.

On the same context, treatment of HepG2 cells with *O. europaea* for 24 hour caused minor increase of cells in sub G1 fraction (6.4 %), and induced cycle arrest in G0/G1 phase (62.8 %). Treatment of HepG2 cells with IC_{50} concentration of *O. europaea* for 48 hour caused increase of cells to 12.3% in sub G peak and slight increase of cells in G//G1 phase by (54.2%), relative to untreated cells. While, treatment with IC_{50} concentration of *O. europaea* for 72 hour caused accumulation of cells by 25.4% in sub G peak.

3.4. Determination of Apoptosis

Untreated HepG2 cells and treated cells were dual stained with propidium iodide (PI) and Annexin V. Untreated HepG2 cells showed presence of unimpaired cells (lower left quadrant), early apoptotic cells (lower right quadrant), late apoptotic or necroptotic cells (upper right quadrant) and primary necrotic cells (upper left quadrant) **(**Figure 3). Treatment of HepG2 cells with IC_{50} concentration of *F. carica* for 24 hour resulted in a statically significant shift of cells (*P* = 0.0001), to early apoptotic cells quadrant and the decrease of cells in the primary necrotic cells quadrant. Moreover, treatment of HepG2 cells with IC_{50} concentration of *F. carica* for 48 and 72 hour resulted in a statically significant shift of cells (*P* = 0.0001), to the early and late apoptotic cells quadrants and decrease of cells in the primary necrotic and live cells quadrants. On the other context, treatment of HepG2 cells with *O. europaea* (olive), for 24, 48 and 72 hour resulted in a statically significant shift of cells **(***P* = 0.0001), to early apoptotic cells quadrant and decrease of cells in the primary necrotic and live cells quadrants.

Table 2: The effect of treatment with *F. carica* (Fig) and *O. europaea* (Olive), leaves extracts on *Cyclin D1, Cdk2* and *Bcl-2* gene expression in Hepatocellular carcinoma cell line (HepG2).

Groups		Relative expression of *Cyclin D1*	Relative expression of *Cdk2*	Relative expression of *Bcl-2*
Control group (Untreated cells)	Min-Max	0.96-0.97	0.75-1.16	1.35-1.36
	Mean ± SD	0.967±0.002	0.9573±0..08	1.34±0.001
Fig24hr.	Min-Max	0.74-0.87	0.649-1.009	1.14-1.27
	Mean ± SD	0.807±0.025	0.8296±0.07	1.20±0.011
Fig48hr.	Min-Max	0.39-0.57	0.613-***0***.657	0.79-0.97
	Mean ± SD	0.484±.035*	0.6351±.0088*	0.88±0.021*
Fig72hr.	Min-Max	0.18-0.32	0.310-***0***.388	0.58-0.72
	Mean ± SD	0.250±0.026*	0.3493±0.015*	0.69±0.023*
Olive24hr.	Min-Max	0.62-0.72	0.534-***0***.660	1.02-1.12
	Mean ± SD	0.672±0.018*	0.5977±0.025*	1.05±0.014*
Olive48hr.	Min-Max	0.52-0.92	0.256-***0***.376	0.65-1.23
	Mean ± SD	0.583±0.17*	0.3549±0.15*	0.85-±0.10*
Olive72hr.	Min-Max	0.44-0.50	0.14-0.226	0.74-0.8
	Mean ± SD	0.47±0.021*	0.206±0.012*	0.77±0.011*

(*) Statistically significant when compared to Control group.
(p) Values were considered significant at level ≤ 0.05.

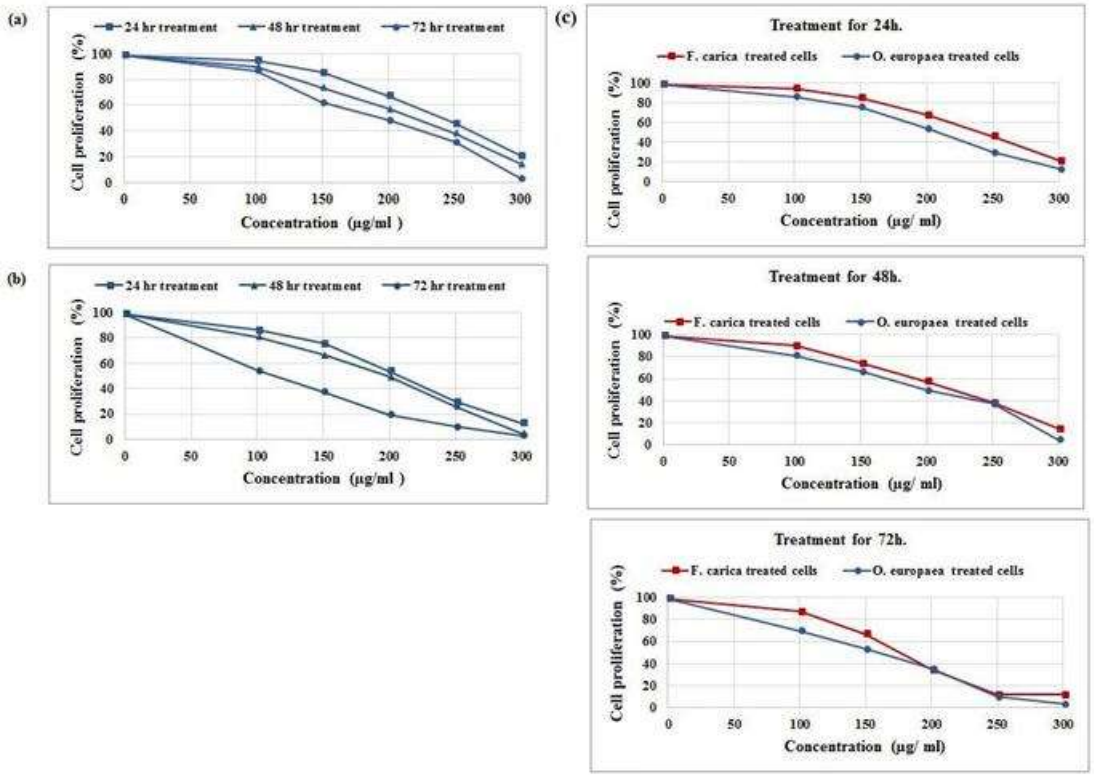

Figure 1: Effect of treatment on the proliferation of HepG2 cells. (a): The effect of treatment with F. *carica* leaves extract on cell proliferation of HepG2 cells after 24,48 and 72 hour of treatment. (b): The effect of treatment with *O. europaea* leaves extract on cell proliferation of HepG2 cells after 24,48 and

72 hour of treatment. (c): Comparison between the effect of treatment with *F. carica* and *O. europaea* leaves extract on cell viability of HepG2 cells after 24, 48 and 72 hour of treatment.

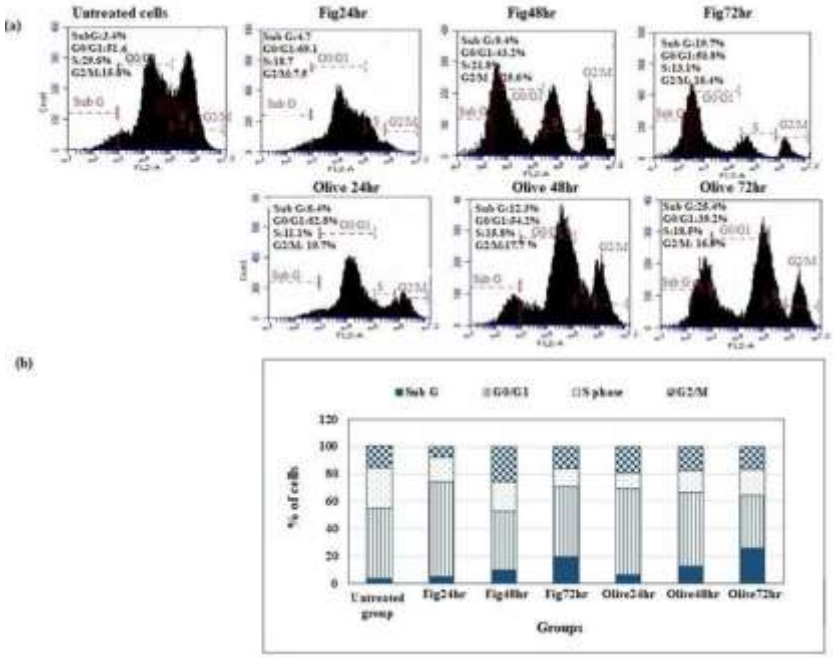

Figure *2*: Effect of treatment with IC_{50} concentrations of *F. carica* and *O. europaea* leaves extract on cell cycle distribution. (a), Histographs of cell cycle distribution of untreated HepG2 and cells treated with *F. carica* and *O. europaea* leaves extract (b), Bar graphs showing the percentage of HepG2 cells in sub G1 population, G0/G1, S, and G2/M phases.

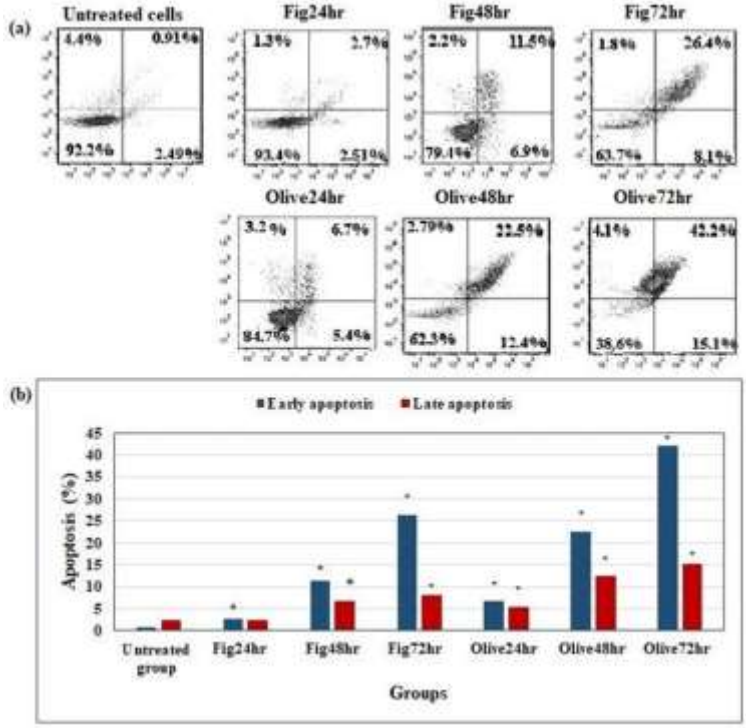

Figure*3*: Effect of treatment with IC_{50} concentrations of *F. carica* and *O. europaea* leaves extract on the induction of apoptosis in HepG2 cell line. (a), Flow cytometry analysis of apoptotic cellular death in HepG2 cells after staining with annexin V and PI. (b), Bar chart representing early and late apoptosis. Significant

results are expressed relative to untreated cells as (asterisks mark *). Statistical difference was considered significant on the level $P < 0.05$. PI, propidium iodide

3.5. Effect of Treatment on *Cyclin D1, Cdk2* and *Bcl-2* Gene Expression

Treatment of HepG2 cells with *F. carica* for 24 hour showed a statically non-significant decrease (*P*=0.16), in the relative expression of *Cyclin D1* relative to untreated control cells. However, increasing the period of treatment for 48 and 72 hour caused a statically significant decrease in *Cyclin D1* levels (*P*=0.0001), compared to untreated cells. In turn, treatment with *O. europaea showed* a statically significant decrease in *Cyclin D1* levels in all periods of treatment

In the same context, treatment with *Ficus carica* leaves extract for 24 hour caused a non-significant decrease (*P*=0.4), in Cdk 2 levels while the statically significant decrease appeared after treatment for 48 and 72 hours (*P*=0.002 and 0.0001; respectively). Alternately, treatment with *Olaea europaea* showed a statically significant decrease in Cdk2 levels after 24,48 and 72 hour of treatment (*P*=0.001, 0.0001 and 0.0001; respectively) (Table 2).

In regard to Bcl-2, gene expression there was a statically non-significant decrease in Bcl-2 levels after treatment with *Ficus carica* leaves extract for 24 hours (*P*=0.32), while the statically significant decrease appeared after treatment for 48 and 72 hours (*P*=0.003 and 0.0001; respectively). Alternately, treatment with Olaea europaea showed a statically significant decrease in Bcl-2 levels after 24, 48 and 72 hour of treatment as (*P*= 0.001, 0.0001and 0.0001; respectively).

DISCUSSION

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and considered as a leading cause of cancer-related death worldwide (Abdel-Malak et al. 2018, Nasar et al. 2019). Tumorigenesis in liver is frequently associated with the existence of lower levels of apoptosis (Puoti, 2018). Moreover, the equilibrium between survival promoting and cell death inducing signals (apoptosis), modulate the initiation and progression of HCC (El-Shafey and Elsherbiny, 2020). The disturbance of equilibrium between these signals caused mainly due to the activation of the anti-apoptotic signals| and the pro-apoptotic molecules (Abdel-Mohsen et al. 2019b; El-Shafey and Elsherbiny, 2020). In the present study, cells cycle distribution of untreated HepG2 cells showed low percentage of apoptotic cells in sub G peak which represent the apoptotic peak (3.4%). Moreover, staining of cells with Annexin V/PI and investigation by flow cytometry displayed low percentage of early and late apoptotic cells. In addition, upregulation of the anti-apoptotic Bcl-2 gene expression in HepG2 cells was detected which may represent development of resistance for apoptosis.

Consequently, development of HCC occurs in a multistep process comprising frequent up-regulation or amplification of cell cycle-associated proteins such as Cyclin D1 and Cdk2 (Sonntag et al. 2018). In normal healthy mammalian cells, Cyclin D1 is involved in the regulation of cell-cycle and is important for the transition of G1/S-phase (Bertoli et al. 2013). It associates with specific cyclin-dependent kinases (Cdks), leading to the phosphorylation of pRB and the stimulation of genes required for G1/Sphase transition and DNA replication (Thwaites et al. 2017). In the present study, the relative expression of cyclin D1 gene expression in HepG2 cells was (0.96). Flow cytometry investigations of HepG2 cells, showed accumulation of cells in G0/G1 and S phases suggesting sustained cellular proliferation. Overexpression of cyclin D1 mRNA and protein, has been established in several solid tumor types, including human parathyroid adenoma, breast, colon, lung and liver cancers. Moreover, it is associated with the early onset of cancer and the aggressive progression of tumor cells (Won et al., 2010). Cyclin D1 is also intimately involved in abnormal cellular proliferation, angiogenesis, and resistance to apoptosis, making it an attractive therapeutic target for reversion of tumor growth (Won et al. 2010; Musgrove et al. 2011).

In the same context, deregulation of cell cycle regulators like Cdks contributes to the tumorigenesis of HCC (Otto and Sicinski, 2017). Cdk2 is an important regulator of eukaryotic cell cycle, which regulate cell proliferation and survival (Huang et al., 2018). Cdk2 regulates the G1/S phase by binding to positive regulators such as cyclin E and cyclin A (Aleem et al., 2004). The Cdk2/cyclin A complex has vital role in the control of S-phase progression, whereas the Cdk2/cyclin E complex is required for the G1 to S-phase transition (Otto and Sicinski, 2017; Aleem et al. 2004). In the extant study, the gene expression of Cdk2 was relatively high (0.975) compared with other groups. It was reported that, the abnormal expression of Cdk2 may lead to uncontrolled cellular growth, which is allied with tumor establishment (Wang et al. 2016). Moreover, its

activity is commonly deregulated in human cancers, resulting in impaired apoptotic cellular death (Wang et al. 2016; Otto and Sicinski, 2017).

Clinical treatment of HCC is still based on traditional methods, such as surgical resection and chemotherapy. Although these methods can ameliorate the prognosis of patients, the effects are unsatisfactory (Daher et al. 2018, El-Abd et al. 2020). Due to increasing drug resistance especially in cancer treatment, plants have become increasingly important in the search for new chemotherapeutic agents. The chemo-preventive and anticancer therapeutic roles exerted by polyphenol plant extracts have formerly been manifested (Mileo and Miccadei, 2016). *Ficus carica* has high polyphenols contents, so far its antioxidant and anti-tumor effects need more biological characterization (Purnamasari et al. 2019). The main phenolic compound found in *F. carica* is quercetin (Hashemzaei, et al. 2017; Purnamasari et al. 2019). Quercetin has the ability to stimulate the apoptosis of colon and leukemia cancer cells by stimulating the emission of cytochrome c from mitochondria (Hashemzaei et al. 2017). In the extant study, treatment of HepG2 cell line with *F. carica* leaves extract caused inhibition of cellular growth in a dose and time dependent manner suggesting its anti-proliferation activity. Additionally, flow cytometry results showed a statically significant increase of cells in early and late apoptotic quadrants suggesting triggering of apoptosis. This was accompanied by the increase of cells in the sub G population (apoptotic peak), and the down-regulation of the anti-apoptotic Bcl-2 gene expression. Furthermore, there were a significant down regulation in the gene expression of the cell cycle regulators (Cyclin D and Cdk2), after 24, 48 and 72 hour of treatment. The ability of *F. carica* to trigger apoptosis could be referred to its flavonoids constituents which activate p53 gene activity and p21 transcription factors leading to binding of Cdk2 with cyclin E and stimulation of cell cycle arrest (Purnamasari et al. 2019; Yee et al. 2015; Vallejo et al. 2012; Toson et al. 2019). Moreover, p53 activation also triggers Bax activity and suppresses Bcl-2 activity which supports our findings. Suppression of Bcl-2 in turn activates caspase cascades leading to triggering of apoptosis (Purnamasari et al. 2019; Vallejo et al. 2012).

Consequently, the potential Biological activity of *O. europaea* is referred to having some phytochemical constituents which utilized for management of numerous diseases (Rahmani et al. 2014). The bioactive constituents of olive leaves extract involved oleuropein and hydroxytyrosol which is the hydrolysis product of oleuropein (Lins et al. 2018). The antioxidant capability of *O. europaea* leaves participates in many therapeutic activities such as chemo-protection, and anti-inflammatory activities (Boss et al. 2016). In regard to the existing study, treatment of HepG2 cells with *O. europaea* leaves extract caused a significant inhibition of cell growth in a dose and time dependent manner suggesting its anti-proliferation activity. Additionally, Flow cytometry results showed a statically significant increase of cells in early and late apoptotic quadrants suggesting triggering of apoptosis. This was accompanied with the increase of cells in the sub G population (apoptotic peak), and the down-regulation of the anti-apoptotic marker (Bcl-2), gene expression. Furthermore, there were a significant reduction in the gene expression levels of the cell cycle regulators Cyclin D and Cdk2 after 24,48 and 72 hour of treatment. This could have referred to the biological activity of Hydroxyty-rosol which mediates the protection of cells from hydrogen peroxide damage, protection of DNA from peroxynitrite-induced damage, blockage of cell cycle progression at the G1 phase and induction of apoptotic cellular death (López et al. 2017).

CONCLUSION

In conclusion, HCC Tumorigenesis involves excessive expression of anti-apoptotic proteins (eg: Bcl-2), and deregulation of the cell cycle machinery. Excessive upregulation of Bcl-2 results in apoptotic resistance. Apoptosis has long been considered as a barrier to carcinogenesis and its induction is crucial to the suppression of tumorigenesis. The present study showed that, *F. carica* and *O. europaea* leaves extracts markedly decreased the proliferation of HepG2 cells. Their antitumor activity was mediated by the down-regulation of the anti-apoptotic Bcl-2 leading to induction of apoptosis. Moreover, these extracts affect negatively on the oncogenic cell cycle regulators (e.g.: cyclin D1 and Cdk -2), leading to their down-regulation and induction of cell cycle arrest. Additionally, they impact positively on the apoptotic machinery leading to triggering of apoptosis and reduction of proliferation. However, the sensitivity of HepG2 cells was higher towards *O. europaea* leaves extract and the best results appeared after 72 hour of treatment.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

SFE and MYN designed the experiment and supervised the experimental work. MSS and SEM performed plant extraction, cell culture, MTT and Real time PCR. ESE and ESE performed flow cytometry experiments, Real time PCR and and data analysis. ESE and ESE wrote and reviewed the manuscript. All authors read and approved the final version.

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